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Butanol is cytotoxic to *Lactococcus lactis* while ethanol and hexanol are cytostatic --Manuscript Draft--

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Abstract:	Lactic acid bacteria currently used extensively by the dairy industry have a superior tolerance towards small chain alcohols, which makes them interesting targets for use in future bio-refineries. The mechanism underlying the alcohol tolerance of lactic acid bacteria has so far received little attention. In the present study the physiological alcohol stress response of <i>Lactococcus lactis</i> subsp. <i>cremoris</i> MG1363 towards the primary, even-chain alcohols; ethanol, butanol, and hexanol was characterized. The alcohol tolerance of <i>L. lactis</i> was found comparable to those reported for highly alcohol resistant lactic acid bacteria. Combined results from alcohol survival rate, live/dead staining, and a novel usage of the beta-galactosidase assay, revealed that while high concentrations of ethanol and hexanol were cytostatic to <i>L. lactis</i> , high concentrations of butanol were cytotoxic, causing irreparable damages to the cell membrane.

1 **Title:** Butanol is cytotoxic to *Lactococcus lactis* while ethanol and hexanol are
2 cytostatic

3 **Running Title:** Butanol cytotoxicity in *Lactococcus lactis*

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ABSTRACT

Lactic acid bacteria currently used extensively by the dairy industry have a superior tolerance towards small chain alcohols, which makes them interesting targets for use in future bio-refineries. The mechanism underlying the alcohol tolerance of lactic acid bacteria has so far received little attention. In the present study the physiological alcohol stress response of *Lactococcus lactis* subsp. *cremoris* MG1363 towards the primary, even-chain alcohols; ethanol, butanol, and hexanol was characterized. The alcohol tolerance of *L. lactis* was found comparable to those reported for highly alcohol resistant lactic acid bacteria. Combined results from alcohol survival rate, live/dead staining, and a novel usage of the beta-galactosidase assay, revealed that while high concentrations of ethanol and hexanol were cytostatic to *L. lactis*, high concentrations of butanol were cytotoxic, causing irreparable damages to the cell membrane.

INTRODUCTION

To forsake modern society's dependency on limited, non-renewable oil-reserves, recent approaches have aimed at constructing microbial hosts for the production of bio-alcohols as alternative fuels. Using several metabolic engineering strategies production of the bio-alcohols ethanol and butanol have been shown exfeasible in a number of organisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Pseudomonas putida*, and *Bacillus subtilis* (1–3).

A major hurdle limiting the yield and titers of microbial bio-alcohol production is the toxicity of these compounds (4). As ethanol and butanol are both short-chain alcohols there are similarities in how microbes respond to them. Through their

47 hydrophobic nature, short chain alcohols disrupt the phospholipid components
48 of the cell membrane thereby increasing membrane fluidity and destroying the
49 integrity of the cell (5–8). A direct correlation between the potency of alcohols as
50 biological effectors and their hydrophobicity has been found (9–11), suggesting
51 that the alcohols primarily influence hydrophobic associations (8). Ethanol
52 stress, the most studied alcohol stress, has been shown to increase membrane
53 permeability allowing for the free passage of ions and charged molecules (8).
54 High concentrations of ethanol and butanol have been shown to interfere with
55 the cell membranes pH gradient thereby increasing acid sensitivity in *S.*
56 *cerevisiae* and *Clostridium* strains, respectively (5,12,13). Introduction of
57 saturated fatty acids have shown to counteract the increased membrane fluidity
58 associated with long term ethanol stress (5,8). In *Lactococcus lactis* subsp.
59 *cremoris* MG1363 an increase in membrane fluidity has likewise been observed
60 as a consequence of sub inhibitory ethanol stress (14). To counteract long term
61 ethanol stress *L. lactis* was found to up-regulate shorter chained fatty acids and
62 down-regulate longer chained unsaturated fatty acids. The shorter chained fatty
63 acid C_{16:1n-7}, known to decrease hydrophobic interaction between the free acyl
64 chains and the proteins, was found up-regulated under prolonged sub inhibitory
65 ethanol stress (14), in accordance with the suggested influence of ethanol on
66 hydrophobic associations (8).

67 In the pursuit of an optimal production strain various organisms have been
68 screened for their alcohol tolerance (15–17), and it has here been found that
69 lactic acid bacteria (LAB) intrinsically possess a superior tolerance towards
70 ethanol compared to other micro-organisms (18,19). Gold *et al.*, determined the

71 ethanol tolerance of 31 *Lactobacillus* strains, and among these 8 showed a
72 significant growth increase in medium containing 16% ethanol (16). In
73 comparison *E. coli* is known to be extremely sensitive towards ethanol with
74 marginal growth in concentrations above 6%, and the obligate ethanol-producer
75 *Zymomonas mobilis* show minimum growth above 9% ethanol (8). In searching
76 for butanol tolerant strains, Knoshaug and Zang determined the butanol
77 tolerance of a range of organisms deemed suitable for production of butanol
78 (15). From a total of 10 *S. cerevisiae* strains, 5 *E. coli*, and 3 *Lactobacillus* strains
79 only two lactobacilli showed an increase in optical cell density in the presence of
80 2.5% n-butanol. One even showed growth in the presence of 3% n-butanol.

81 Although the superior alcohol tolerance of LAB have long been known (8,19) no
82 studies have characterized the mechanism behind LAB's superior alcohol
83 tolerance. *L. lactis* subsp. *cremoris* MG1363 represents a suitable model
84 organism for the analysis of alcohol tolerance in LAB because; (i) it has a simple
85 fermentative metabolism in which glucose is converted almost exclusively into
86 lactic acid (20), (ii) a wide range of regulatory networks has been characterized
87 easing physiological and metabolic understanding (21,22), (iii) the genome
88 sequence is known (23), and lastly, (iv) a large array of genetic tools are
89 available (24–26). As a case study for its use as a biofuel producer, *L. Lactis*'s
90 ethanol production pathway was changed from lactic acid to ethanol, reaching
91 yields comparable to those reported for optimized *S. cerevisiae* strains (27).

92

93 In the present study, novel insight into the alcohol tolerance of LAB is achieved
94 through characterization of growth physiology, cell survival, and membrane

integrity of *Lactococcus lactis* subsp. *cremoris* MG1363 when grown in the presence of the short primary alcohols ethanol, butanol, and hexanol.

METHODS

Strains and culture conditions: *Lactococcus lactis* subsp. *cremoris* MG1363, a plasmid-free derivative of *L. lactis* subsp. *cremoris* NCD0712 (28) was used throughout. PRJ4621 is a derivative of MG1363 containing the plasmid pAK80 with the synthetic promoter CP25 in front of *lacLM* from *Leuconostoc lactis* (29).

L. lactis were grown in the chemically defined SA medium (30) modified by exclusion of acetate and supplemented with 2 $\mu\text{g ml}^{-1}$ lipoic acid and 1% glucose (GSAL) and incubated at 30°C without aeration but with slow stirring to ensure homogeneity. Medium with excess buffering capacity was achieved by supplementing with twice the amount of the pH buffer morpholinepropanesulfonic acid (MOPS) as in regular GSAL (GSAL^{MOPS}).

To ensure statistical significance all growth experiments were performed with a minimum of three biological replicas.

Alcohol stress response: The alcohol stress response of *L. lactis* was determined by adding exogenous alcohol to cultures that had grown exponentially for at least ten generations, ensuring a balanced pre-stress phenotype. By exposing growing cultures ($\text{OD}_{450} \sim 0.4 \text{ ml}^{-1}$) to a range of ethanol (25 – 115 mg ml^{-1}), butanol (5 – 25 mg ml^{-1}), or hexanol (1 – 3 mg ml^{-1}) concentrations the tolerance range of *L. lactis* towards the specific alcohols was determined. The alcohol stress response was quantified through growth rates (h^{-1}), final cell density

yields (OD₄₅₀), and pH. Amount of product produced and substrate consumed was quantified by HPLC following overnight stress inoculation.

Quantification of extracellular glucose and end products: To determine the extracellular metabolite levels in the growth medium, culture samples were filtrated through a 0.22 µm filter. Glucose, lactate, acetate, formate, and ethanol were separated using an Ultimate 3000 high-pressure liquid chromatography system (Dionex, Sunnyvale, CA) equipped with an Aminex HPX-87H column heated (Bio-Rad, Hercules, CA) and a Shodex RI-101 detector (Showa Denko KK, Tokoy, Japan). The column temperature was set to 60°C and 5mM H₂SO₄ was used a mobile phase with a flow rate of 0.6 ml min⁻¹.

Acid sensitivity assay: Cultures grown in GSAL or in GSAL^{MOPS} were subjected to a range of exogenous ethanol (25 – 115 mg ml⁻¹), butanol (5 – 20 mg ml⁻¹), and hexanol (1 – 3 mg ml⁻¹) concentrations. Differences between final pH's and between final yields of alcohol-stressed cultures grown in GSAL^{MOPS} to alcohol-stressed cultures grown in GSAL were used to quantify acid sensitivity.

Survival rate under alcohol stress: The survival rate of *L. lactis* when exposed to the individual alcohols was determined by measuring the survival in colony forming units (CFU) after 120 min of alcohol stress. Concentrations used; ethanol (55 – 120 mg ml⁻¹), butanol (10 – 25 mg ml⁻¹), and hexanol (2 – 3 mg ml⁻¹). Aliquots were removed at 0 (reference), 10, 20, 40, 80, and 120 min after induction of alcohol stress, and the first six serial dilutions were plated on GM17 agar and incubated at 30°C over night (31). The relative increase in CFU compared to the reference was calculated.

141 *Quantification of the Live/dead ratio:* The live/dead ratio of alcohol stressed *L.*
142 *lactis* was determined in-situ using the BacLight Live/dead Bacterial Viability
143 Kits, L7012, provided by Molecular Probes and imaged using Zeiss Axioplan
144 Fluorescence Microscope with a RS Photometrics CoolSNAP Camera. The filter
145 had an excitation of 450-490 nm with long pass 520 nm enabling simultaneously
146 visualization of SYTO 9 and propidium iodide dye. Exponentially growing *L. lactis*
147 was harvested and re-suspended in cold GSAL medium to yield a final OD₄₅₀ ~
148 1.5. Aliquots of the harvested cells were exposed to ethanol, butanol, or hexanol
149 for 5 min on ice before being mixed with the staining agents SYTO 9 and
150 propidium iodide dye and visualized by microscopy. A minimum of 3 samples of
151 at least 500 fluorescent cells each were counted for each condition and used to
152 determine the live/dead ratio. For further details regarding the Live/dead kit
153 used see the manufactures product manual, Molecular Probes (Live/dead
154 BacLight Bacterial Viability Kit:
155 <https://tools.lifetechnologies.com/content/sfs/manuals/mp07007.pdf>).

156 *Assay of cell permeability towards ONPG.* The amount of ortho-nitro
157 phenylgalactoside (ONPG) passing the membrane of PRJ4621 cells was
158 measured as the rate of ortho-nitro phenol (ONP) formation inside the cells. The
159 strain PJR4621 has a high constitutive β -Galactosidase activity, and the rate of
160 ONP formation was determined using the standard protocol described by Miller
161 with the following modifications (32). Exponentially growing cells OD₄₅₀ ~ 0.4
162 ml⁻¹ were either harvested for β -Galactosidase assay or incubated at 30°C for 1
163 hour with ethanol (120 mg ml⁻¹), butanol (25 mg ml⁻¹), or hexanol (2.5 mg ml⁻¹).
164 The non-stressed harvested cells, were washed and lysed with chloroform, left

untreated or subjected to either ethanol (120 mg ml⁻¹), butanol (25 mg ml⁻¹), or hexanol (3 mg ml⁻¹) for 10 min at 30°C prior to addition of ONPG and subsequent quantification of β -Galactosidase activity. The alcohol stressed cells were harvested and washed followed by treatment with chloroform or left untreated for 10 min at 30°C. ONPG was hereafter added and the rate of ONP formation was quantified.

RESULTS AND DISCUSSION

Tolerance of *L. lactis* towards primary alcohols categorizes *L. lactis* as a highly butanol tolerant strain

The tolerance range of *Lactococcus lactis* subsp. *cremoris* MG1363 against the primary alcohols ethanol, butanol, and hexanol was determined by exposing exponentially growing *L. lactis* to a broad range of exogenous alcohol concentrations (Fig. 1). All growth experiments were performed in the chemically defined SA medium (30) supplemented with 1% glucose (GSAL), to avoid evoking the purine sensitive phenotype of *L. lactis* (33). Fig. 1a, 1b, and 1c shows the growth curves obtained for the first 100 min after addition of increasing concentrations of ethanol, butanol, or hexanol. The specific growth rates obtained under alcohol stress as a function of the final alcohol concentration in the growth medium, revealed, as expected, an inverse correlation between growth rate and alcohol concentration (Fig. 1d, 1e, and 1f). As a measure of the tolerance of *L. lactis* towards the individual alcohols, the alcohol concentration that resulted in 50% decrease in the specific growth rate was determined. This was observed for ethanol at 55 mg ml⁻¹, butanol at 10 mg ml⁻¹, and hexanol at 1.5 mg ml⁻¹, corresponding to (7%, 1.2%, and 0.2% (v/v),

respectively). At these concentrations a constant flux of glucose consumption could be detected (data not shown) confirming that the vitality of the bacteria was intact.

When the final optical density was plotted as a function of alcohol concentrations (Fig. 2a, 2b, and 2c) a 50% decrease in final yield was observed for ethanol, butanol, and hexanol at 75, 15, and 2.5 mg ml⁻¹, corresponding to 10%, 2%, and 0.3% (v/v), respectively, indicating a high level of alcohol resistance.

Alcohol does not induce increased acid sensitivity

To assess if alcohol stress causes increased acid sensitivity in *L. lactis*, a growth-based assay was devised, in which the buffering capacity of the medium could be increased by doubling the concentration of the buffering agent. This assay was used to distinguish between two contrasting models; one where the alcohol stress causes an increased acid sensitivity (model 1), or one where the alcohol stress does not cause an increased acid sensitivity (model 2). If the final OD was determined by a pH threshold (model 1), then a doubling of the buffering capacity of the medium would result in a doubling of the final OD of the culture reaching the same pH threshold. If, in contrast, the final OD was determined by other alcohol induced thresholds (model 2), then a doubling of the buffering capacity would have no affect on the final yield, but would result in a higher final pH.

In Fig. 3a, 3b, and 3c a set of growth experiments has been represented in two types of plots. The first type of plots shows the increase in final pH upon doubling the buffering capacity of the medium (left axis) as a function of alcohol

213 concentration. Since unstressed cultures use the excess buffering capacity to
214 increase biomass production and continue fermentation until the normal pH
215 threshold is reached, doubling the buffering capacity only insignificantly
216 increased the pH level (0.1 pH unit), as shown in Fig. 3a, 3b, and 3c for the
217 unstressed conditions (0 mg ml⁻¹). The excess biomass production upon
218 doubling the buffering capacity of the medium (right axis) is shown as a function
219 of alcohol concentration in the second type of plots. Plots from all three alcohol
220 stress conditions showed that higher alcohol concentrations follow model 2,
221 since the increase in biomass yield is reduced to zero and the final pH is elevated.
222 Butanol at 15 mg ml⁻¹ has the most severe effect on the increase in final pH (1.6
223 pH units).

224 Growth cessation was not caused by sugar starvation, as an excess of glucose
225 was found to be present in the medium (data not shown). Furthermore, the
226 higher pH observed at higher alcohol concentrations was not accompanied by a
227 shift in the general metabolism since the cultures performed homolactic
228 fermentation throughout the experiment (data not shown).

229 **Butanol is cytotoxic while ethanol and hexanol is cytostatic to *L. lactis***

230 Alcohol could be either cytostatic or cytotoxic to the lactococcal cell. If alcohol is
231 cytostatic it inhibits cell growth as long as the stress is applied, but the inhibition
232 is reversed when the stress disappears. In contrary, if alcohol is cytotoxic, the
233 inhibition is not reversed. The survival rate of *L. lactis* exposed to the three
234 alcohols was determined by quantifying the number of colony forming units
235 (CFU) at various time points before and after the onset of alcohol stress. Only
236 alcohol concentrations that had been found to inhibit growth to less than half-

237 maximal growth rates were used. Fig. 4a, 4b, and 4c show the growth in optical
238 density before and after addition of ethanol, butanol, or hexanol, respectively.
239 Fig. 4d, 4e, and 4f show the relative CFU determination (survival) of *L. lactis*
240 exposed to ethanol, butanol, or hexanol.

241 Interestingly, it could be noted from all curves in Fig. 4d, 4e, and 4f that even
242 though a steady increase in OD₄₅₀ was observed for all of the tested alcohol
243 concentrations (Fig. 4a, 4b, and 4c), none of the cultures showed any significant
244 increase in CFU. Cell clumping and chain formation are known to underestimate
245 the number of CFU under heat stress of *L. lactis* (34), and phase-contrast
246 microscopy revealed that *L. lactis* forms chains when dividing during ethanol,
247 butanol, and hexanol stress (data not shown). As shown in Fig. 4e, high butanol
248 concentrations caused severe cell death with a fast death rate at a concentration
249 of 25 mg ml⁻¹. In contrast, high concentrations of ethanol (Fig. 4d), and hexanol
250 (Fig. 4f) did not lead to cells death, suggesting that butanol is cytotoxic to *L. lactis*
251 whereas both ethanol and hexanol are cytostatic. It should be noted that no
252 decrease in optical density was observed for butanol treated *L. lactis* cells, thus
253 butanol is not suspected to cause cell lysis.

254 To substantiate the claim that high butanol concentrations are cytostatic, the
255 cytotoxicity of the three alcohols was investigated *in situ* using live/dead
256 staining. Live/dead staining uses a double labeling technique with the green-
257 fluorescent SYTO 9 and the red-fluorescent propidium iodide (PI). PI is
258 frequently used in lactococcal research to distinguish dead from living cells, as
259 only cells with irreparable damaged membranes are stained (35–37), while
260 SYTO 9 stains the DNA of both living and dead cells. Exponentially growing cells

261 in GSAL medium were harvested and subjected to ethanol, butanol, or hexanol at
262 120, 25, or 3 mg ml⁻¹ for 5 min on ice, respectively, before addition of the
263 live/dead stain. Fluorescence microscopy imaging was subsequently used to
264 determine the ratio of dead vs. live cells (Fig. 5).

265 By inspection of the staining pattern in Fig. 5, it became clear that the ratio of
266 dead cells was higher after butanol addition than after addition of ethanol or
267 hexanol, in accordance with the results from the survival determination.
268 Quantification of the live/dead-ratio of *L. lactis* showed that ethanol at 120 mg
269 ml⁻¹ and hexanol at 3 mg ml⁻¹ slightly lowered the percentage of live cells from
270 100% in untreated cells to 86% ± 8% and 91% ± 2%, respectively. In contrast,
271 butanol at 25 mg ml⁻¹ decreased the fraction of living cells to 35% ± 8%. Even
272 when the butanol concentration was reduced to 21 mg ml⁻¹, a low survival rate
273 was observed (25% ± 17%), confirming that high butanol concentrations are
274 toxic towards *L. lactis*.

275 To confirm the observation that higher concentrations of butanol causes damage
276 to the cell membrane, the ability of ortho-nitro phenyl galactoside (ONPG) to
277 cross the membrane under ethanol, butanol, and hexanol stress was determined.
278 An *L. lactis* strain PRJ4621, with a high constitutive β-Galactosidase activity, was
279 used for detection of intracellular ONPG by conversion to ONP. Chloroform is
280 usually used to permeabilize cells prior to determination of β-Galactosidase
281 activity in bacteria (32), and Fig. 6 shows that non-permeabilized cells produce
282 very little ONP. Exposure to chloroform for 10 min resulted in high ONP
283 production (1000 miller units). Treatment with ethanol at 120 mg ml⁻¹ for 10
284 min had no effect, while exposure to butanol or hexanol for 10 min (at 25 mg ml⁻¹

285 and 2.5 mg ml⁻¹, respectively) resulted in permeabilization close to that of
286 chloroform (Fig. 6). The obtained β -Galactosidase activity in PRJ4625 was
287 comparable to those observed in the literature (29).

288 Surprisingly, both butanol and hexanol caused permeabilization of the
289 membrane towards ONPG, since only butanol was found to be cytotoxic. It was
290 therefore hypothesized that the damage by hexanol could be reversible and thus
291 cytostatic, while the damage by butanol was irreversible leading to cytotoxicity.
292 To test this, exponentially growing cells of PRJ4621 were first subjected to
293 ethanol, butanol, or hexanol (120, 25, or 2.5 mg ml⁻¹ respectively) for 1 hour. The
294 stressed cells were then harvested, washed, and assayed for production of ONP.
295 The maximal ONP production rate was found for each condition by assaying a
296 chloroform permeabilized sample. The relative ONP production rate between
297 untreated and chloroform permeabilized cells for each stress condition is shown
298 in Fig. 7. For unstressed cells the intracellular ONP production rate was
299 approximately 60-fold elevated by chloroform permeabilization. In contrast, for
300 butanol treated cells the chloroform permeabilization only result in 5-fold
301 elevation of the ONP production rate, showing that the cells are already highly
302 permeable to ONPG. It is evident from Fig. 7 that the hexanol permeabilization is
303 partially reversed, since chloroform is needed for ONPG to enter the cell. The 28-
304 fold elevated ONP production between chloroform treated and non-treated
305 hexanol stressed cells is almost half of the observed 59-fold change for the
306 control condition, which supports the suggestion that butanol is cytotoxic
307 because it creates irreversible damage to the membrane that results in increased
308 permeability to small impermeable molecules. Due to the irreversible nature of

butanol induced membrane disruption, it could be hypothesized that butanol forms stable pores in the membrane, while the larger hexanol form chains that align with the phospholipids. Upon removing hexanol stress, the aligned hexanol chains may distribute evenly resulting in a partial reversal of the permeabilization.

CONCLUSION

Combined results from the alcohol survival rate, live/dead staining through SYTO 9 and PI, and novel usage of the β -galactosidase assay, revealed that while high concentrations of ethanol and hexanol were cytostatic to *L. lactis*, high concentrations of butanol were cytotoxic through disruption of the cell membrane.

For the future industrial use of LAB as butanol producers, the cytotoxic effect of butanol needs to be addressed before selecting production strains. In this regard, *L. lactis* is unsuitable unless the cytotoxicity of butanol is diminished. Adaptive laboratory evolution has been initiated to provide butanol resistant strains for the further characterization of a *L. lactis* strain with improved butanol tolerance.

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Figure 1: Stress response of *L. lactis* towards exogenous ethanol, butanol, and hexanol. (a-c) Growth of *L. lactis* in GSAL medium in the presence of various concentrations of (a) ethanol, (b) butanol, and (c) hexanol was monitored by OD₄₅₀. Exogenous alcohol was added in mid-exponential phase (OD₄₅₀ ~0.4 ml⁻¹). (d-f) Growth rates, μ (h⁻¹), were calculated from the growth curves (a-c) and plotted against the final concentrations of (d) ethanol, (e) butanol, and (f) hexanol. (a) Concentrations of ethanol in mg ml⁻¹: 25 [filled circles], 55 [open circles], 75 [filled squares], 95 [open squares], and 115 [filled triangles]. (b) Concentrations of butanol in mg ml⁻¹: 5 [filled circles], 10 [open circles], 15 [filled squares], 20 [open squares], and 25 [filled triangles]. (c) Concentrations of hexanol in mg ml⁻¹: 1.0 [filled circles], 1.5 [open circles], 2.0 [filled squares], 2.5 [open squares], and 3.0 [filled triangles]

Figure 2: Final yield as defined by OD₄₅₀ of *L. lactis* grown in GSAL as a function of increased ethanol (a), butanol (b), and hexanol (c) concentrations. Exogenous alcohol was added in mid-exponential phase (OD₄₅₀ ~0.4 ml⁻¹), and final yield was measured after overnight incubation.

Figure 3: Acid sensitivity assay of alcohol stressed *L. lactis*. Exogenous alcohol was added in mid-exponential phase (OD₄₅₀ ~0.4 ml⁻¹). Final yield, as defined by OD₄₅₀, and pH were measured after overnight incubation of *L. lactis* grown in regular GSAL medium and in GSAL with doubled buffering capacity. Left axis, relative increase in final pH [filled circles]; and right axis, relative increase in final yield [open circles] by doubling the buffering capacity of the medium as a function of increased concentrations of ethanol (a), butanol (b), and hexanol (c).

Figure 4: Relative survival of *L. lactis* grown in GSAL after addition of ethanol (a, d), butanol (b, e), or hexanol (c, f). Exogenous alcohol was added in mid-exponential phase (OD₄₅₀ ~0.4 ml⁻¹, reference time point). Relative survival was determined by measuring changes in colony forming units (cfu) following the onset of stress as compared to the reference condition. (a, d) Concentrations of ethanol in mg ml⁻¹: 55 [filled circles], 75 [open circles], 95 [filled squares], and 120 [open squares]. (b, e) Concentrations of butanol in mg ml⁻¹: 10 [filled circles], 20 [open circles], 22.5 [filled squares], and 25 [open squares]. (c, f) Concentrations of hexanol in mg ml⁻¹: 2.0 [filled circles], 2.5 [open circles], 2.75 [filled squares], and 3.0 [open squares].

Figure 5: In situ live/dead imaging of *L. lactis* (a), or *L. lactis* exposed to 120 mg ml⁻¹ of ethanol (b), 25 mg ml⁻¹ of butanol (c), or 3 mg ml⁻¹ of hexanol (d). Exponential growing cells was harvested and subjected to ethanol, butanol, or hexanol stress before staining with SYTO 9 and propidium iodide. Cells with physically intact membranes are shown in green while cells with damaged membranes are shown in red.

Figure 6: Intracellular production of ONP from ONPG in a Lac⁺ derivative of *L. lactis*. Exponentially growing cells of a *L. lactis* Lac⁺ derivative (PRJ4621) were harvested and permeabilized with chloroform, ethanol (120 mg ml⁻¹), butanol (25 mg ml⁻¹), or hexanol (2.5 mg ml⁻¹) following by determination of ONP production from ONPG.

Figure 7: Relative ONP production rate in a Lac⁺ derivative of *L. lactis*. Exponentially growing cells of a *L. lactis* Lac⁺ derivative (PRJ4621) were subjected to either non-stressed, ethanol (120 mg ml⁻¹), butanol (25 mg ml⁻¹), or hexanol (2.5 mg ml⁻¹) stress for one hour following which the cells were either permeabilized with chloroform or not, and ONP production from ONPG was determined. The relative difference in ONP production between the chloroform permeabilized and non-permeabilized cells is shown.

Fig. 1

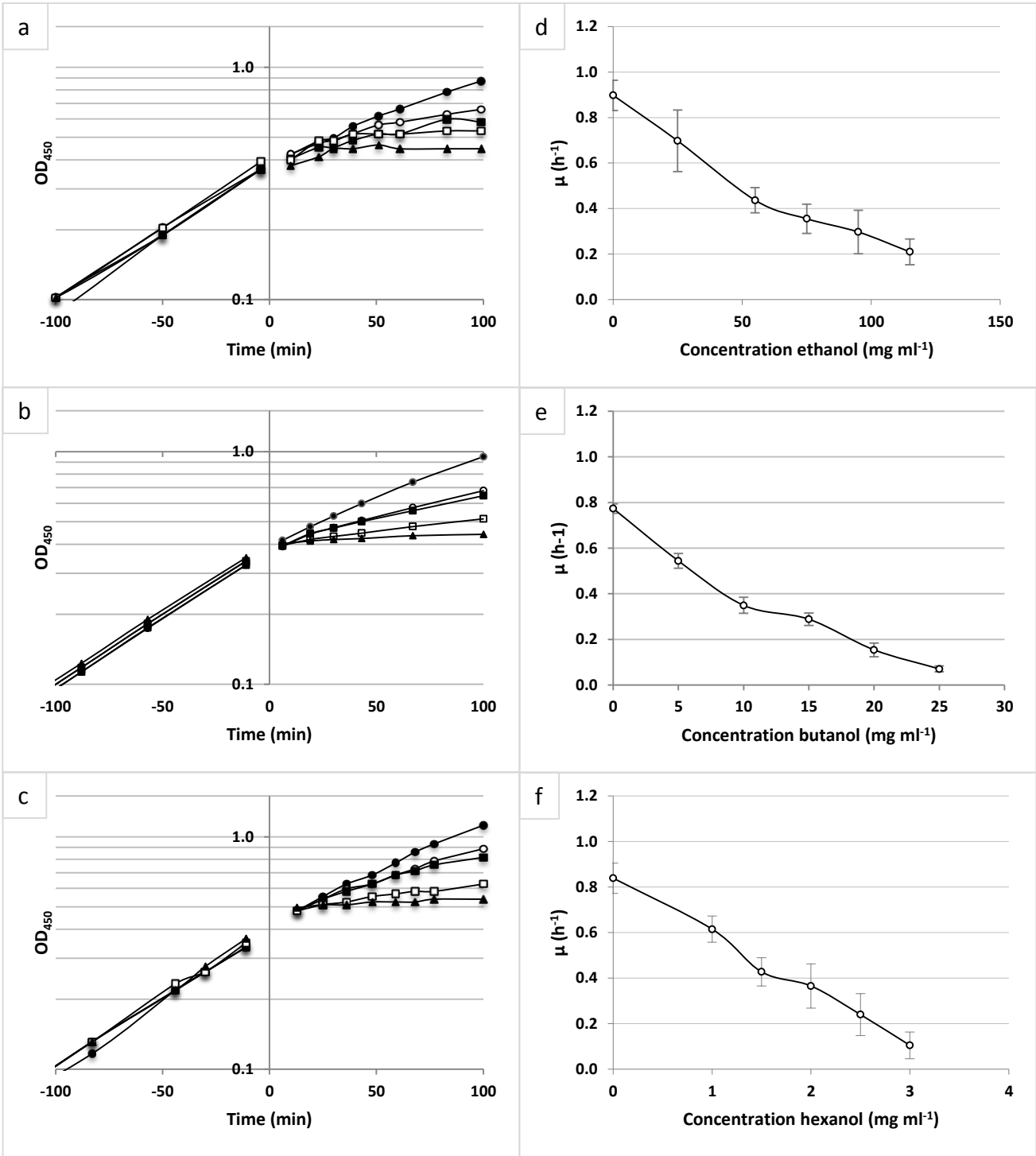


Fig. 2

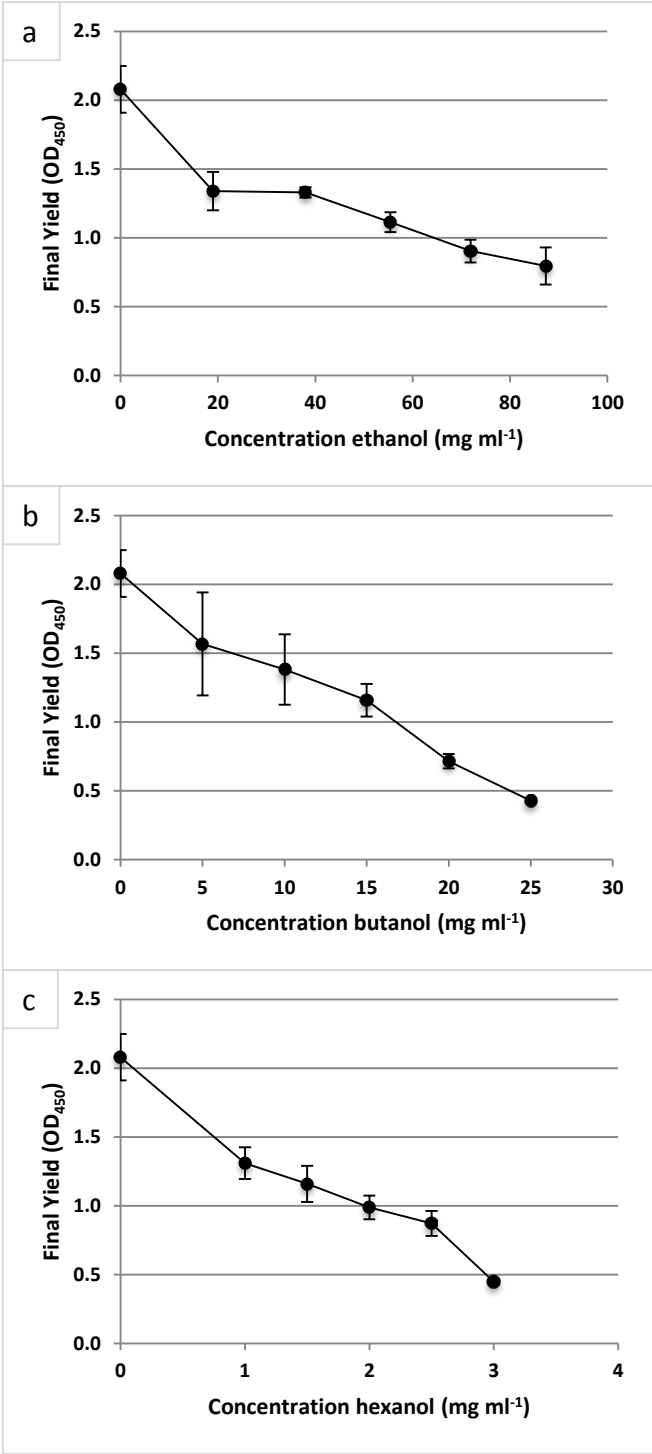


Fig. 3

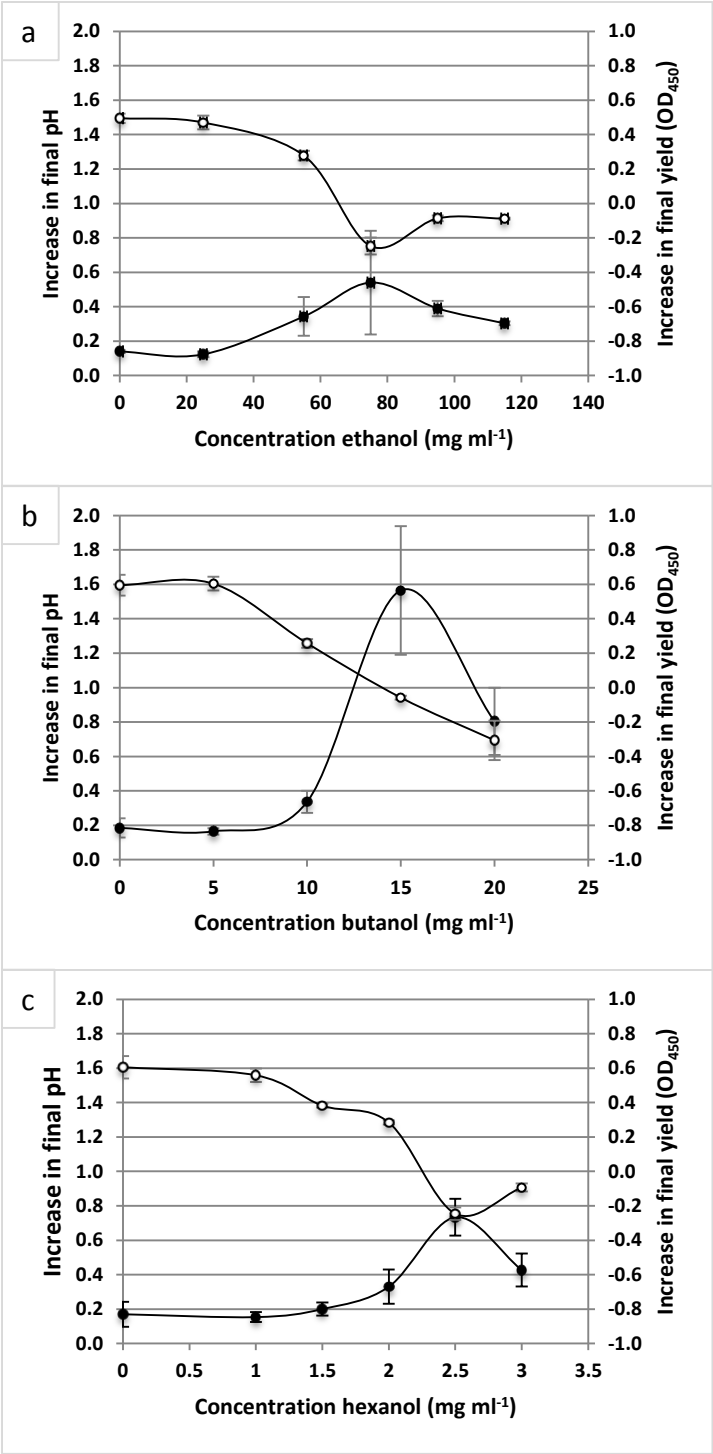


Fig. 4

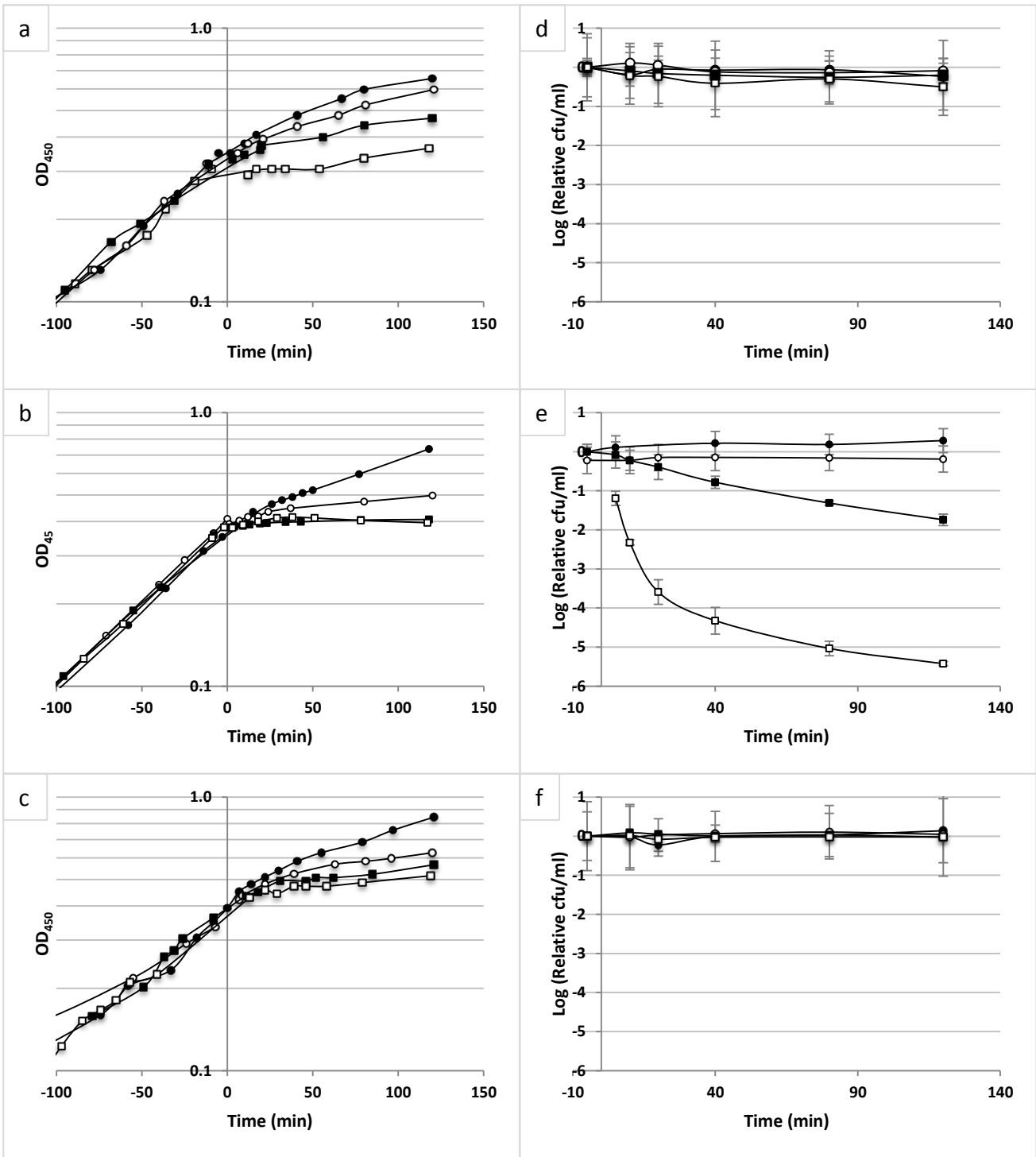


Fig. 5

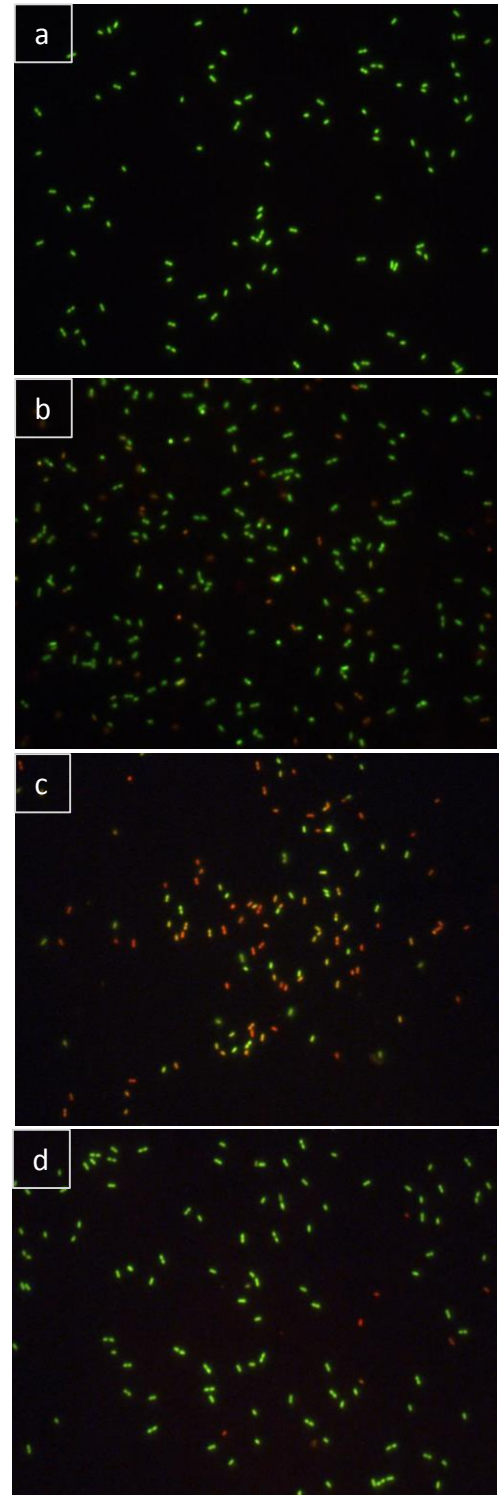


Fig. 6

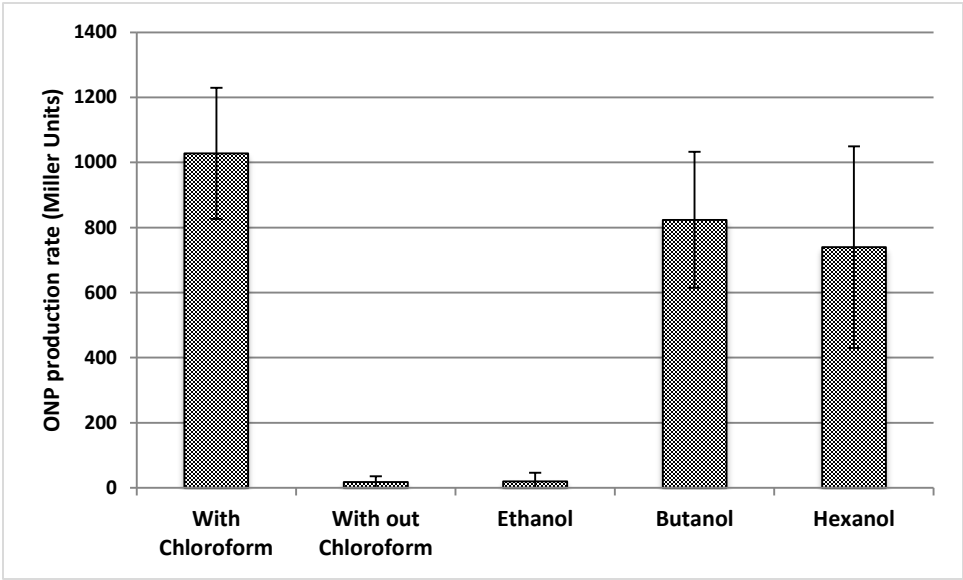


Fig. 7

